AD-A065 576 BIORESEARCH INC FARMINGDALE NY F/G 6/13
DEVELOPMENT OF A METHODOLOGY FOR THE RAPID DETECTION OF COLIFOR--ETC(U)
MAY 78 A M CUNDELL, A M PISANI, E FINDL N00600-77-C-1163
UNCLASSIFIED ER-9011-4 DTNSRDC/SME-78-56 NL

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DEVELOPMENT OF A METHODOLOGY FOR THE RAPID DETECTION OF COLIFORM BACTERIA

DTNSRDC REPORT SME-78-56

MAY 1978

Prepared under:
Contract N-00600-77-C-1163

Prepared for:
David W. Taylor Naval Ship Research
and Development Center
Annapolis, Md. 21402

Prepared by:
BioResearch, Inc.
315 Smith Street
Farmingdale, NY 11735

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DAVID W. TAYLOR NAVAL SHIP RESEARCH
AND DEVELOPMENT CENTER

HEADQUARTERS
BETHESDA, MARYLAND 20084

ANNAPOLIS LABORATORY
ANNAPOLIS, MD 21402

CARDEROCK LABORATORY
BETHESDA, MD 20084

IN REPLY REFER TO:
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26 FEB 1979

From: Commander, David W. Taylor Naval Ship R&D Center
To: Chief of Naval Material (MAT 08T241)

Subj: Development of a Methodology for the Rapid Detection of Coliform Bacteria

Encl: (1) DTNSRDC Report SME-78-56

1. The Environmental Protection Agency in 1976 specified that by 1980 liquid discharge from human waste sources contain no more than 200 fecal coliform (E. Coli) per 100 mL. Current analytical techniques, Most Probable Number (MPN) and Membrane Filter (MF), require 72 hours and 24 hours, respectively, to complete the analysis. The long analytical time prohibits close monitoring of waste water treatment devices. Therefore, a research investigation was conducted to develop a technique for rapid (approximately one hour) measurement of coliform bacteria in the laboratory. Enclosure (1) is a report of the investigation which was jointly funded by the Navy and Army (David W. Taylor Naval Ship R&D Center; Naval Construction Battalion Center, Civil Engineering Laboratory; Office of Naval Research; US Army Mobility Equipment R&D Command; and US Army Medical Bioengineering R&D Laboratory).

2. The technique developed and reduced to practice during this investigation is based on the ability of live coliform bacteria to synthesize an enzyme that decomposes lactose or a structurally similar compound. In the procedure developed, such a compound is used to induce the bacteria to synthesize that enzyme. This is followed by diffusing into the bacteria another compound containing fluorescein which is chemically bound to a lactose structure. When so bound, the fluorescein will not fluoresce. However, when the enzyme synthesized by the bacteria hydrolyzes that compound, the fluorescein is released and will fluoresce under incident light. After induction of enzyme synthesis, the effluent sample to be analyzed is atomized into microdroplets which are suspended in silicone oil on a microscope slide. Individual bacteria can be detected and counted in this manner with a fluorescence microscope. A good correlation between the percentage of fluorescent droplets in random fields of view and the cell density in the bacterial suspension has been established in the range of 10^7 to 10^{10} E. Coli per 100 mL. Possible methods of concentrating suspensions of coliform bacteria were reviewed

and a procedure based on co-centrifugation of the coliforms with excess numbers of Staphylococcus epidermidis was developed. Coliform concentration up to 1000 times was achieved using a fixed angle centrifuge, lowering the detection limit to 10^4 E. Coli per 100 mL.

3. Based on the above results, investigation is continuing to (a) confirm the feasibility of the methodology using a wide variety of wild coliforms, (b) confirm the feasibility of detecting and quantifying coliform at the 200 per 100 mL level, and (c) develop an instrumentation system for automating the detection of coliforms. A technique involving liquid membrane micro-encapsulation of the sample, plus flow cell counting of fluorescent bacterial microbubbles contained in the sample will also be investigated. If successful, this technique would eliminate the need for concentration in order to reach a detection level of 200 coliforms per 100 mL.

4. Funding for continuation of the work is being provided by the Office of Naval Research and US Army Mobility Equipment R&D Command.

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1. REPORT NUMBER: SME-78-56	2. GOVT ACCESSION NO.
3. RECIPIENT'S CATALOG NUMBER	
4. TITLE (and Subtitle): (6) DEVELOPMENT OF A METHODOLOGY FOR THE RAPID DETECTION OF COLIFORM BACTERIA.	5. TYPE OF REPORT & PERIOD COVERED: Research & Development 9-30-77 / 5-30-78-Final Rpt.
	6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s): (10) A.M. Cundell, A.M. Pisani, E. Findl	8. CONTRACT OR GRANT NUMBER(s): (15) N-00600-77-C-1163
9. PERFORMING ORGANIZATION NAME AND ADDRESS: BioResearch, Inc. 315 Smith Street Farmingdale, NY 11735	10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS: Program Element 62765N Task Area ZF-57-572-003 Work Unit 2860-101
11. CONTROLLING OFFICE NAME AND ADDRESS: David W. Taylor Naval Ship R&D Center Annapolis, Maryland 21402	12. REPORT DATE: (11) May 1978
	13. NUMBER OF PAGES: 37 (12) 40p.
14. MONITORING AGENCY NAME & ADDRESS(if different from Controlling Office): (9) Final rept. 30 Sep 77 - 30 May 78,	15. SECURITY CLASS. (of this report): Unclassified
	15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

Unlimited.

DISTRIBUTION STATEMENT A
Approved for public release;
Distribution Unlimited

(16) F57572

(17) ZF57572003

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

(14) ER-9011-4

18. SUPPLEMENTARY NOTES

DDC RECEIVED MAR 12 1979 B

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

Coliform Bacteria
β-D-galactosidase
Fluorescence Detection
Coliform Detection Technique

S/C 393778

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

A technique is described for the detection and quantification of coliform bacteria in less than 1-1/2 hours. The detection method is based on the presence of β-D-galactosidase activity within this class of bacteria.

The biochemical reactions exploited in the BioResearch rapid detection method are (1) induction of β-galactosidase within E. coli by the inducer isopropyl thio β-D-galacto-pyranoside (IPTG), (2) transport of the fluorescein-conjugated

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SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

79 03 01 022

substrate fluorescein di β-D-galactopyranoside (FDG) into the bacterial cells and (3) hydrolysis of the substrate to liberate the fluorescent dye fluorescein. This is followed by the detection of the fluorescein dye, whose absorption and fluorescence spectra are well known.

FOREWORD

This report was prepared by BioResearch, Inc. for the David W. Taylor Naval Ship R and D Center (DTNSRDC), Annapolis, MD. in accordance with the requirements of Contract No. N-00600-77-C-1163. The period of performance of the contract was September 30, 1977 to May 30, 1978. The objective of the program was the determination of the feasibility of a fluorescence technique for the rapid detection of coliform bacteria.

In addition to the David W. Taylor Naval Ship R and D Center, the following agencies participated in funding the research program; (1) U.S. Army Mobility Equipment Research and Development Command (USMERADCOM), Ft. Belvoir, VA, (2) U.S. Army Medical Bioengineering Research and Development Laboratory (USAMBRDL), Ft. Detrick, MD (3) Naval Civil Engineering Laboratory (CEL), Port Hueneme, CA. and (4) Office of Naval Research (ONR), Arlington, VA.

All measurements and calculations contained herein are expressed in SI units.

The program was carried out by the Bioelectrochemistry Division of BioResearch, Inc. Dr. A. M. Cundell, Manager of the Microbiology Department was the technical manager of the program. He was assisted by A. M. Pisani. Technical support was provided by E. Findl.

The principal technical monitor for the program was Mr. Lynne Harris (DTNSRDC). He was assisted by Mr. Dan Lent (MERADCOM) and Dr. Howard Bausum (USAMBRDL).

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GLOSSARY

Coliform bacteria: Aerobic and facultative anaerobic gram-negative, non-spore forming, rod-shaped bacteria that ferment lactose, with gas formation, within 48 hours at 35°C, e.g., Salmonella spp., Shigella spp. and Escherichia coli. They inhabit the lower intestine of warm-blooded animals.

Escherichia coli: The most well known coliform bacterium.

Escherichia coli Neotype: The strain of E. coli designated as the culture maintained in culture collections as the representative of the organism for taxonomic purposes. The selection of a neotype usually implies that the organism is physiologically typical of the species.

β-D-galactosidase: The inducible enzyme that hydrolyzes lactose to glucose and galactose.

Staphylococcus epidermidis: A gram-positive coccus that lacks the enzyme β-d-galactosidase. A non-pathogenic inhabitant of the human skin.

FDG: Fluorescein di β-D-galactopyranoside. Fluorogenic substrate for β-D-galactosidase.

IPTG: Isopropyl thio β-D-galactopyranoside. Inducer of β-D-galactosidase.

ONPG: O·Nitrophenol galactopyranoside. Colorimetric substrate for β-D-galactosidase.

MPN: Most Probable Number determination. Statistical method for estimating the cell density of coliform bacteria in the multiple-tube fermentation technique.

ABSTRACT

A rapid (on the order of one hour) method for the detection of coliform bacteria, based on β-D-galactosidase activity, has been investigated. This method involves the induction of the enzyme in Escherichia coli Neotype using Isopropyl thio β-D-galactose (IPTG). A cell suspension of the induced organisms is dispersed onto a modified microscope slide containing silicone oil, with an aerosol sprayer. Within the resultant microdroplets, a fluorogenic substrate, fluorescein-di β-D-galactopyranoside, is hydrolyzed by the coliform bacteria. The liberated fluorescein is concentrated within individual oil-encpasulated droplets, enabling them to be visualized and counted with a fluorescence microscope. A good correlation between the percentage of fluorescent droplets in random fields of view and the cell density in the bacterial suspension has been established, in the range of 10^5 to 10^8 E. coli per ml. To calibrate the technique against a standard method for coliforms, the number of E. coli in samples were counted using both the Most Probable Number, multiple tube fermentation technique and plate counts.

A comparison was made between the number of coliforms determined by the rapid coliform detection method, plate counts on Eosin Methylene Blue and MacConkey's agars, multiple tube fermentation and membrane filter techniques. The samples investigated were a cell suspension of E. coli Neotype and primary-treated sewage.

Possible methods of concentrating suspensions of coliform bacteria were reviewed and a procedure based on co-centrifugation of the coliforms with excess numbers of Staphylococcus epidermidis was developed. Coliform concentration up to 1,000 times was achieved using a fixed-angle centrifuge.

INTRODUCTION

Rapid determination of the level of coliform bacteria contamination has been a goal of microbiologists for many decades. The term rapid seems to have different meanings to various investigators trying to develop techniques to overcome the problem. As defined herein, rapid detection of coliform bacteria means detection and quantification of the first sample in less than two (2) hours of elapsed time.

A preliminary study of the problem of rapid coliform detection was carried out under U.S. Navy Contract N-00167-76-M-8206. That study detailed most of the methodologies listed in the literature up to mid 1976. One technique, involving enzyme mediated fluorescence, was uncovered that appeared to offer promise of being specific to the detection of coliform bacteria at the 2 per ml level in less than two (2) hours. [This is the 1980 Environmental Protection Agency (EPA) discharge requirement, as specified in the Federal Register Vol. 41, #71, Page 15, 324, Monday, April 12, 1976.] Further, it appeared that the technique could be made specific to fecal coliform bacteria as well.

As a result of the preliminary study, five DOD centers, i.e., David W. Taylor Naval Ship R&D Center (DTNSRDC), U. S. Army Mobility Equipment Research and Development Command (USMERADCOM) U. S. Army Medical Bioengineering Research and Development Laboratory (USAMBRDL), Naval Civil Engineering Laboratory (CEL) and Office of Naval Research (ONR) funded a study to evaluate the feasibility of the enzyme fluorescence technique. Results of this feasibility study are described herein.

TECHNICAL DISCUSSION

Detection of coliform bacteria within a water sample is universally accepted as an indication of fecal contamination of that sample. Coliform bacteria, especially Escherichia coli, are used as indicators of fecal pollution in preference to human bacterial pathogens or other intestinal bacteria because:

1) pathogenic bacteria may be emitted intermittently or in small numbers,

2) pathogenic bacteria may be fastidious in their growth requirements, hence difficult to culture,

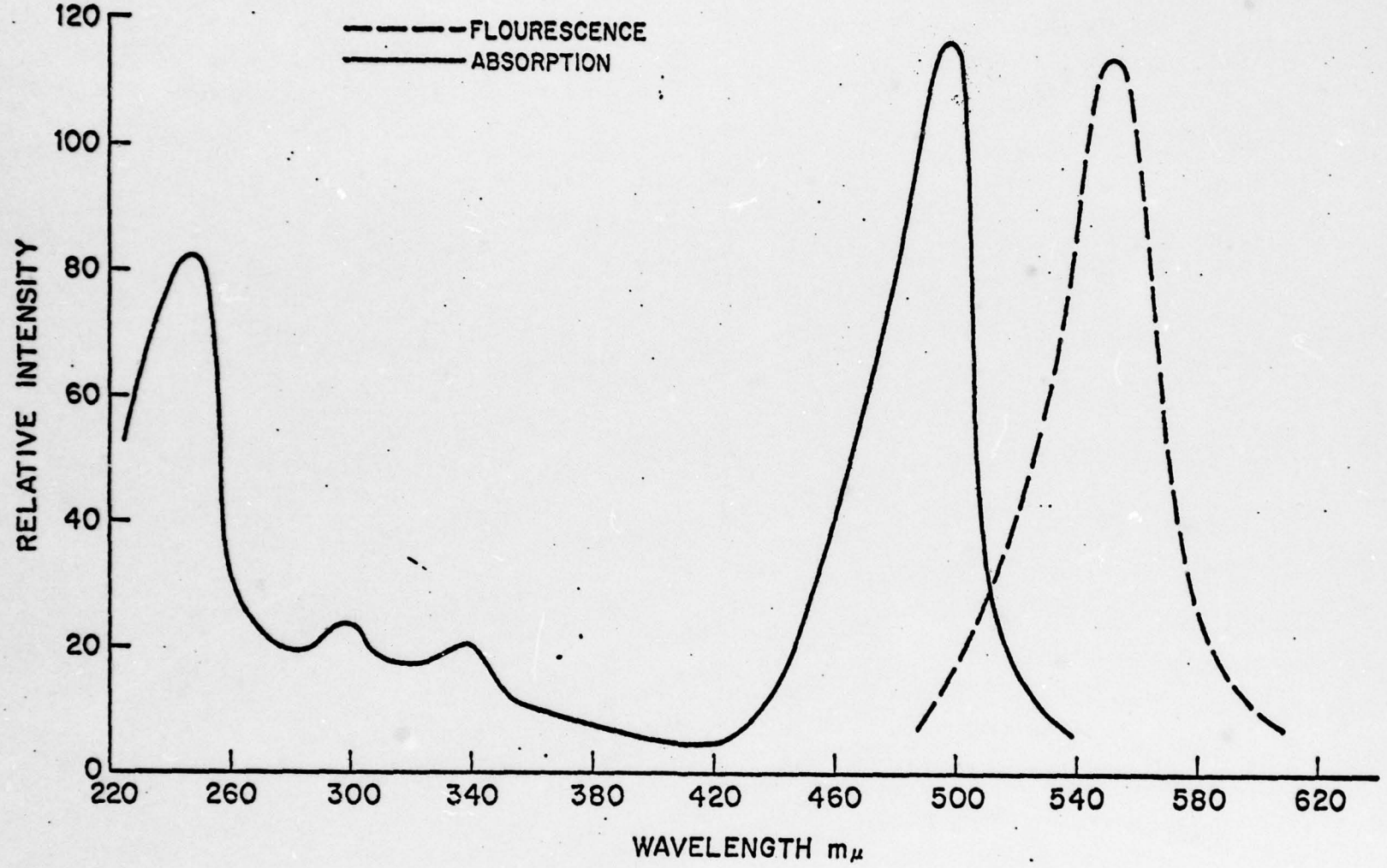
3) E. coli are present in greater numbers than other intestinal organisms that are readily cultured,

4) E. coli survive longer in the environment outside the human intestine, but do not usually multiply (1) (2).

Coliform bacteria are defined as aerobic and facultative anaerobic gram-negative, non-spore forming, rod-shaped bacteria that ferment lactose, with gas formation, within 48 hours, at 35° C. (3) It follows from the operational definition of coliform bacteria that they will possess the enzyme B-galactosidase, which is required to ferment lactose. This enzyme is synthesized by the bacterium in response to the presence in the environment of an inducer, which is usually lactose or some structurally related chemical compound. (The classical work on the induction of enzyme synthesis by Pardee, Jacob and Monod (4) was concerned with the enzyme B-galactosidase.) A coliform detection method, based on the presence of B-galactosidase activity within bacteria, should therefore be feasible.

The biochemical reactions exploited in the Bio-Research rapid detection method are 1) induction of B-galactosidase within E. coli by the inducer isopropyl thio B-D-galactopyranoside (IPTG), 2) transport of the fluorescein-conjugated substrate fluorescein di B-D-galactopyranoside (FDG) into the bacterial cells and 3) hydrolysis of the substrate to liberate the fluorescent dye fluorescein. This is followed by the detection of the fluorescein dye, whose absorption and fluorescence spectra are illustrated on Figure 1.

The minimum number of molecules of fluorescein that can be detected, based upon a conservative 10^{-5} moles per liter detection limit and a 10^{-12} liter droplet size,



FLUORESCENCE AND ABSORPTION SPECTRUM
OF FLUORESCEIN Na.

FIGURE 1

can be estimated using the following relationship.

$$n = A \alpha V$$

where n = minimum number of fluorescein molecules required for detection

A = 6.023×10^{23} molecules per mole (Avogadro's number)

α = detection limit (moles per liter)

V = volume of sample (liters)

hence, n = 6.023×10^{6} molecules

The time required to form 6.023×10^{6} molecules of fluorescein can be estimated from the turnover rate of the enzyme β-D-galactosidase, i.e., the number of molecules of lactose hydrolyzed per second, and a knowledge of the average number of enzyme molecules per bacterium.

$$t = n/\phi \cdot \rho$$

where t = time required

ϕ = turnover of β-D-galactosidase (120 molecules per second)

ρ = number of molecules of enzyme per fully induced bacterium (500)

hence, t = 100 seconds.

[In actuality, transport of the fluorescent substrate into the bacterium via the permease system and the diffusion of free fluorescein from the bacterium may be the limiting factors.]

General Procedures Used

The procedure adopted for the rapid detection of coliform bacteria, is based on the pioneering work of Dr. Boris Rotman [5] of Brown University, who developed techniques for the detection of single molecules of

β-D-galactosidase from E. coli. Steps involved in the procedure are as follows:

1. Uninduced E. coli $\xrightarrow[\text{Lactate}]{\text{IPTG}}$ Induced E. coli

2. $FDG_{(outside)} \xrightarrow{\text{Permease}} FDG_{(inside)}$

3. FDG $\xrightarrow{\text{β-D-galactosidase}}$ Fluorescein + galactose

4. $Fluorescein_{(inside)} \longrightarrow Fluorescein_{(outside)}$

5. Excitation + $Fluorescein_{(outside)} \rightarrow$ Fluorescein emission

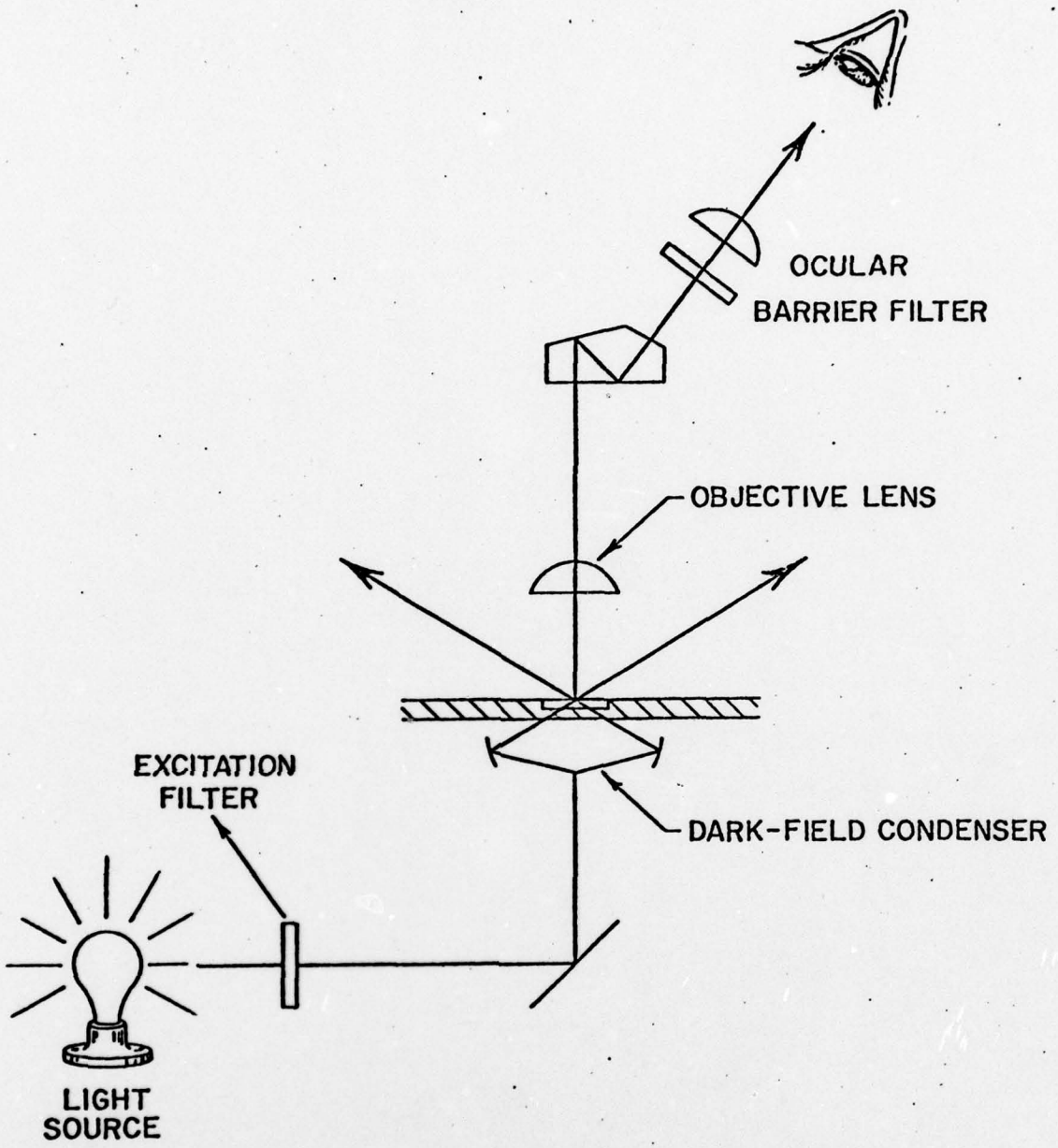
Although fluorescein is highly fluorescent and can be detected at low concentrations, the amounts of fluorescein that can be produced by a single induced bacterium will, under normal circumstances, be rapidly diluted by the water surrounding the bacterium. Rotman solved the dilution problem by containing one (or a small number) of E. coli within microdroplets of water sprayed into and surrounded by silicone oil. Since fluorescein and water are essentially insoluble in the silicone oil used, dilution of the fluorescein released by the coliform bacterium is limited to the small volume of the microdroplet. [Microdroplet volumes are typically 10 to 30 x 10^{-9} cm^3.]

Diffusion of FDG into the bacterial cells and diffusion of the liberated fluorescein out can be accelerated by treating the cell suspension with isoamyl alcohol to perforate the cell membrane.

Instrumentation

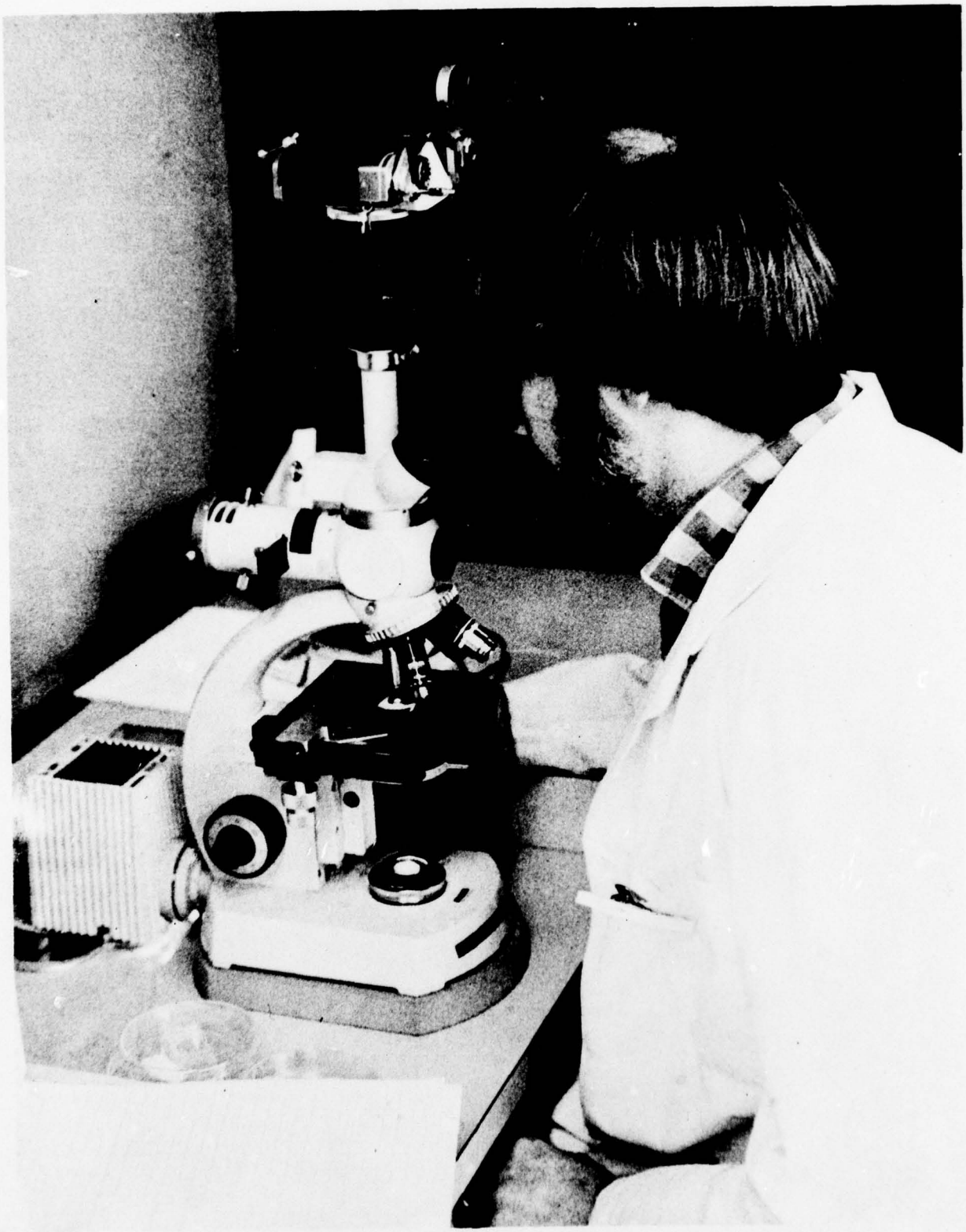
Fluorescence Microscope

A Zeiss Standard 15 microscope, equipped for transmittance fluorescent light observation was used in this investigation. The source of illumination was a 12 volt Halogen-quartz light source, with a BG 38 excitation filter and a 510 barrier filter. The light source was focused on the microscope slide by an oil immersion, darkfield condenser. Optics used were a 10X, Achromat objective lens and paired KPL 12.5X eyepieces. (See Figures 2 and 3.)



SCHEMATIC REPRESENTATION OF THE FLUORSCENCE MICROSCOPE USED IN THE RAPID COLIFORM DETECTION PROCEDURE

FIGURE 2



ZEISS STANDARD 15 MICROSCOPE
TEST SETUP
FIGURE 3

Aerosol Spray and Modified Microscope Slides

Droplets (diameter 5-30 μm) are produced by a simple atomizer operated by N_2 gas delivered at 13.8KPa (2Psi) (Figure 4). Tapered capillary tubes for the atomizer are constructed from pyrex melting point tubes (outside diameter 1.5 mm, wall thickness 0.25 mm). Ten centimeter lengths are placed in a Narishige PE-2 electrode puller and extruded (magnet setting 3.5, heater setting 4.0). The tip diameter is checked with a microscope (brightfield 625X magnification) and tapered capillary tubes, with tip diameters below 20 μm, are cut and selected for use in the atomizer.

Darkfield slides are washed in acetone and coated with silicone oil (Siliclad #J-600). Parafilm is cut to cover the slide and a chamber in the sheet of parafilm, the size of a coverslip, is cut out. The slide and parafilm are warmed on a hot plate to seal the parafilm to the slide. The parafilm chamber is filled with 3-4 drops of silicone oil (SF-96(50)) and the slide placed on a strip of indicator paper (pH 5.0).

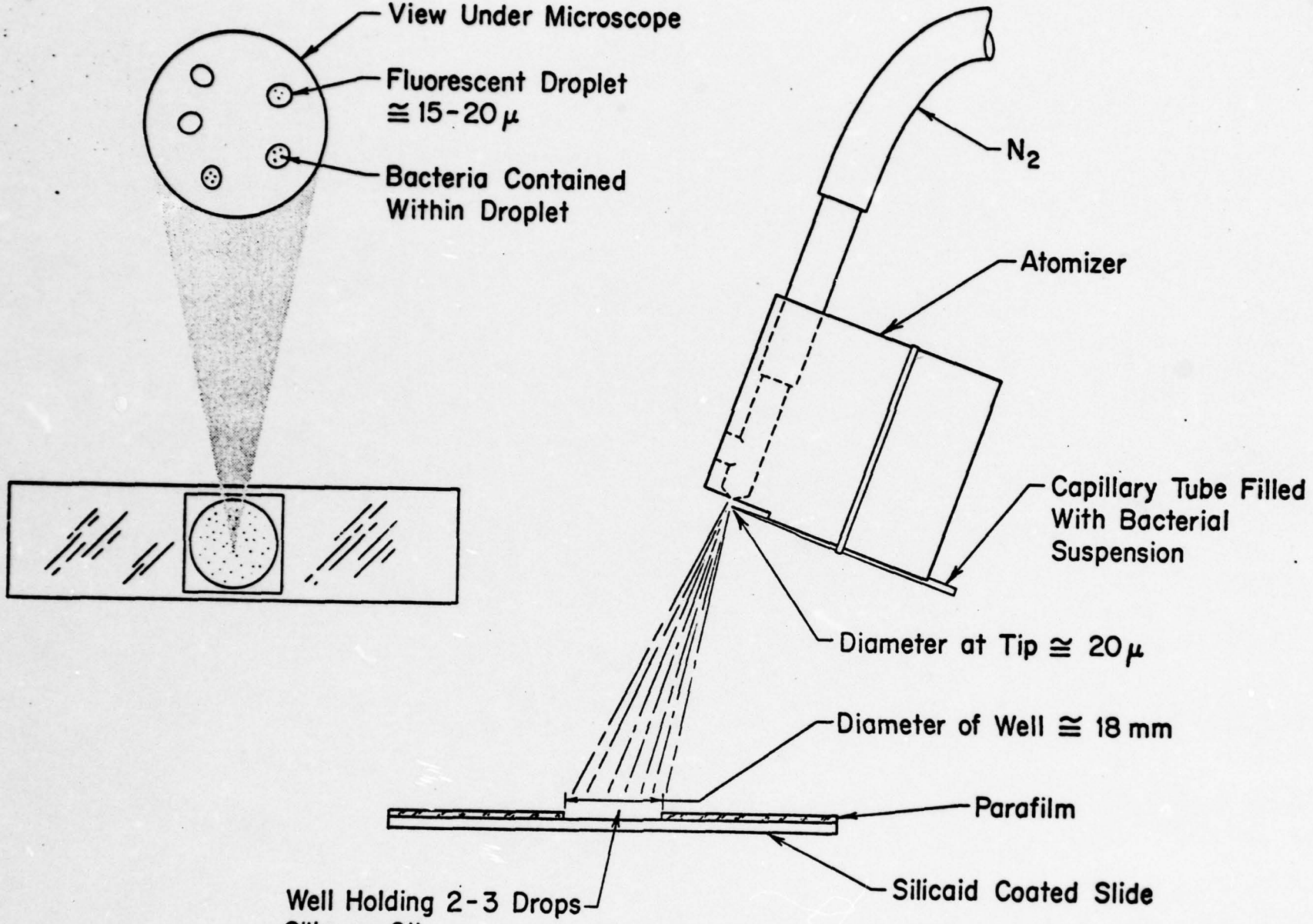
The surface of the slide is sprayed with the bacterial suspension using an atomizer. Spray characteristics are checked by observing the color change of the indicator paper. A drop of silicone oil is placed on the coverslip and the chamber closed. The slide is incubated for 15 minutes at 35°C. If necessary, the slide can be centrifuged to expedite the settlement of the droplet.

Reagent Preparation

Reagents used for bacterial culturing and enzyme analysis are listed below, along with their preparation procedures.

1) Lactate Medium

K_2HPO4	6.968 g
KH_2PO_4	4.082 g
$(NH_4)_2\ SO_4$	1.004 g
$MgSO_4\ 7H_2O$	0.098 g
Distilled Water	1000 ml
Casamino Acids	40 μg/ml
Sodium Lactate	0.4%



SCHEMATIC DRAWING OF SPRAYING PROCESS

FIGURE 4

2) Induction Medium

Isopropyl thio β-D-galactopyranoside (IPTG)

2×10^{-4} M in Lactate broth.

3) Phosphate Buffer

Dissolve 34.0 g of KH_2PO_4 in 500 ml of distilled water, adjust pH to 7.2 with 1N NaOH, add 1.25 ml of the above stock and 5.0 ml of 5% $MgSO_4 \cdot 7H_2O$ to 1000 ml of distilled water.

4) Fluorescein-conjugated substrate fluorescein-di(β-D-galactopyranoside) (FDG) 2×10^{-3}M in Lactate medium.

The FDG solution, as received from the manufacturer, was found to be too impure for use. This was confirmed by spectrophotometric analysis of the as-received material. A sharp peak at ≈ 490 mμ indicated that fluorescein was the principal contaminant.

The following paper chromatographic technique was used to separate the FDG from the fluorescein. Ten (10) mg of FDG was dissolved in a minimal amount of distilled water and loaded onto the origin of a 20 x 20 cm. sheet of Whatman #1 chromatography paper. A descending mode was used, with the top layer of a 1-pentanol: 1-propanol: water (40:11:15) mixture as the solvent for up to four days at room temperature. After this period of time, the FDG moves one to two cm. from origin and can be separated from fluorescein, which moves further from the origin. Location of the FDG is determined by cutting narrow strips from each edge of the paper and exposing them to an ultraviolet light source (chromatography paper containing FDG will fluoresce). The section of paper containing FDG is dried at room temperature. It is then eluted with distilled water. The amount of FDG in the eluate can be measured by the abosrbance of the solution at 224nm wavelength in a UV spectrophotometer. [Fifty (50) units of optical density corresponds to a 7.6×10^{-4}M solution.] The solution is then sterilized by Millipore filtration and stored in a freezer at -20°C.

An enzyme assay is used to determine the concentration of FDG in a purified solution. The following reagents are mixed and used in the FDG assay.

0.2 ml of FDG solution

0.2 ml of 0.2 M $NaPO_4$ buffer, pH 7.2

0.02 ml of enzyme

The mixture is incubated for 4 hours at 37°C. To it is added 1.6 ml of water and 0.01 ml of 5N NaOH. Concentration is measured using the optical density of the solution at 490 mμ. (Specific absorbance is 1.36 for 1×10^{-3} M FDG.)

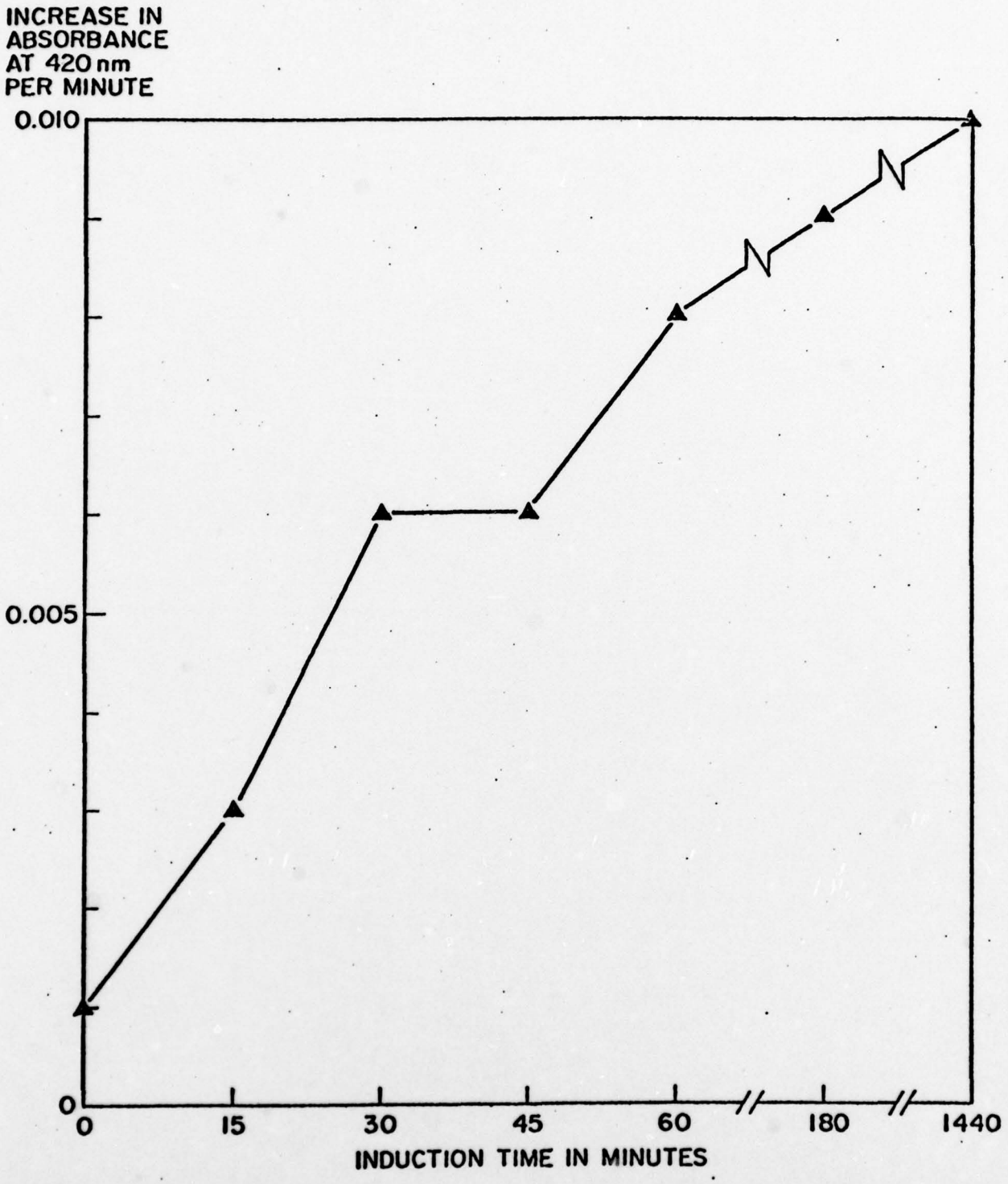
Induction of β-D-galactosidase Activity

The inducer IPTG was used at a concentration of 2×10^{-4}M for the induction of sufficient β-D-galactosidase activity in the E. coli for rapid FDG hydrolysis. An incubation time for the induction was determined by measuring the initial rate of o-nitrophenol-galactose (ONPG) hydrolysis by an E. coli suspension containing 10^8 cells per ml, incubated at 35°C for 0, 15, 30, 45, 60, 180 minutes and 24 hours. ONPG hydrolysis (3×10^{-3}M in phosphate buffer) was measured in a Beckman Spectrophotometer by following the increased absorbance at 420 nM for 5 minutes. A plot of the increase in absorbance per minute against induction time (Figure 5), indicated that a 30 minute induction period was sufficient to achieve an increased β-D-galactosidase activity within the bacterial cells.

Test Procedure

E. coli Neotype (ATCC # 11775) was grown in a lactate medium supplemented with casamino acids. The lactate medium was loop inoculated with the E. coli from a nutrient agar slant, incubated in a rotatory incubator and maintained at 35°C for 12 to 18 hours. Cells, grown overnight on lactate broth, were refreshed by being diluted with broth (1 to 3) and returned to a 35°C incubator, so that the organisms would reenter the log growth phase. Cells were harvested by centrifugation, resuspended in lactate broth containing 2×10^{-4}M IPTG, and incubated for 30 minutes to induce sufficient β-galactosidase activity for rapid FDG hydrolysis.

Induced cell suspensions were centrifuged to remove IPTG and resuspended in lactate broth at cell densities ranging from 10^5 to 10^8 organisms per ml.



INITIAL RATE OF ONPG HYDROLYSIS BY A E.coli SUSPENSION (10^8 cells per ml) INDUCED WITH 10^{-4}M IPTG

FIGURE 5

To 200 µl of the bacterial suspension was added 25 µl of a 7.4×10^{-4}M FDG solution (final concentration 1.5×10^{-4}M). The mixture was stored on ice until the slide was sprayed with the suspension. The surface of the silicone oil in the chamber was sprayed with bacterial suspension from a height of about 5 cm using an atomizer with a 10-20 µm diameter tapered capillary tube. (See Figure 6).

After spraying, the chamber was sealed with a coverslip. FDG hydrolysis was expedited by treating the cell suspension with isoamyl alcohol prior to spraying, (0.2 ml of isoamyl alcohol to 1 ml of cell suspension) to increase permeability of the bacterial cell wall.

Sprayed slides were incubated at 35°C for 15 minutes and then examined using a fluorescence microscope. Single coliforms contained within the sprayed microdroplets are readily visualized. Droplets containing 2 coliforms have 2x the fluorescence of a single coliform, 3 coliforms 3x and so on. The best visualization of the fluorescent droplets was obtained using a BG 38 excitation filter and a 510 nM barrier filter in the left eyepiece. Using this technique, fluorescent droplets are superimposed on the darkfield image. On each slide, 10 fields of view, having a diameter of 700 µm, were selected at random, using the low power objective lens (10X magnification). The total number of droplets and fluorescent droplets in the 15-20 µm diameter size range was counted in each field. In addition, the cell density within each cell suspension was determined by plate counts on nutrient agar.

Using the techniques outlined above, three replicate determinations were run for five different coliform densities between 10^5 and 10^8 bacteria per ml.

Test Results

A plot of the percentage of fluorescent droplets per field of view against the cell density of the E. coli Neotype determined by plate counts is shown on Figure 7. It appears to be linear in the 10^6 to 5×10^8 range (correlation coefficient = 0.76; line of best fit $y = 2.93X - 38.0$, estimate of the good of fit $t = 2.86^*$, df = 23). (Details of the statistical analyses are presented in the Appendix). As shown on Figure 7, quantification to the 10^5 coliforms/ml is feasible. Each determination took about 80 minutes to complete. This time was divided into the dilution of E. coli suspension (2 minutes), IPTG induction (30 minutes), centrifugation and resuspension (10 minutes), addition of FDG and isoamyl alcohol (2 minutes), spraying (1 minute), incubation of the slide (15 minutes) and counting of 10 fields of view on each slide (20 minutes).

* Significant at the 5% level

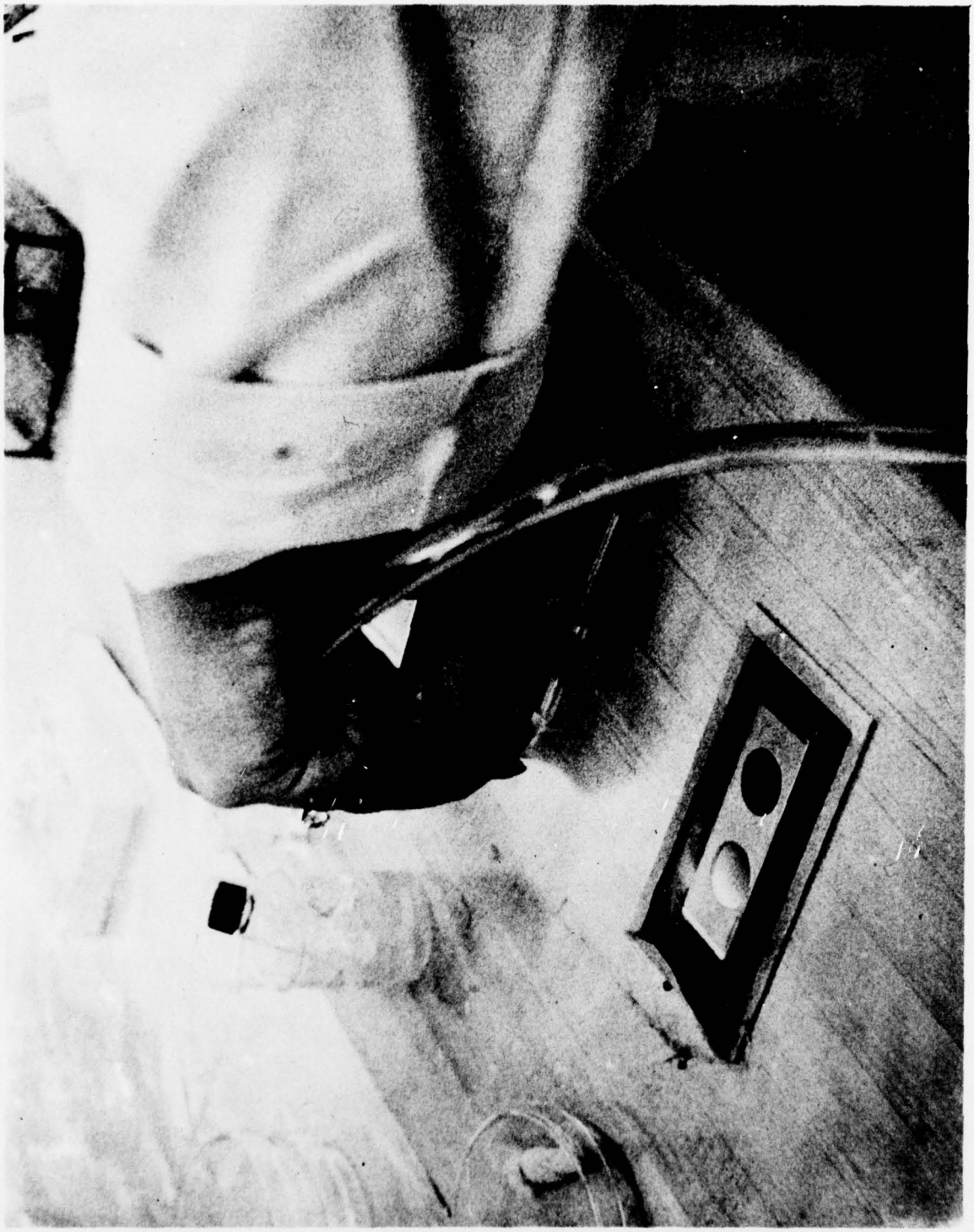
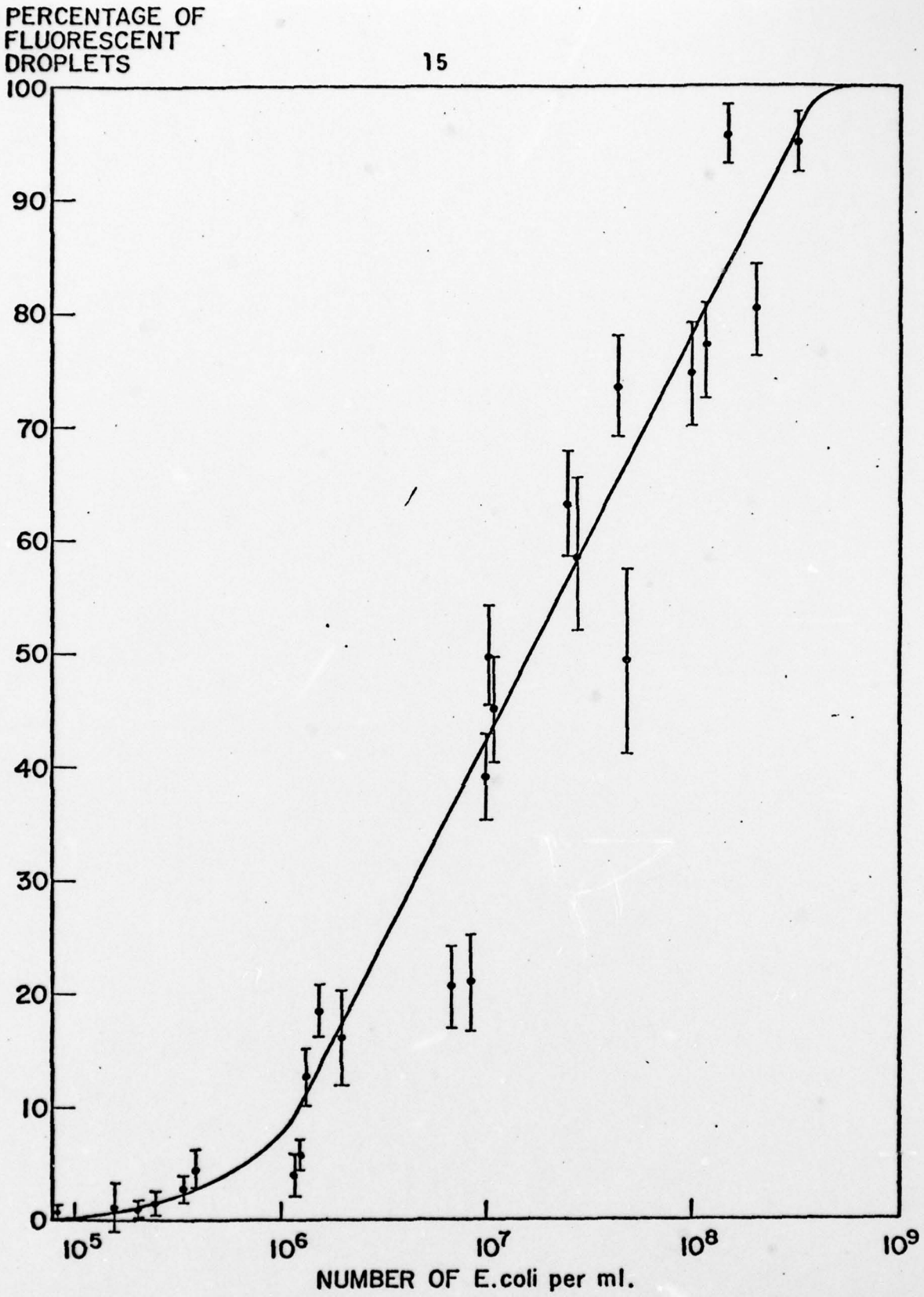


FIGURE 6 - PHOTOGRAPH OF SPRAYING PROCESS



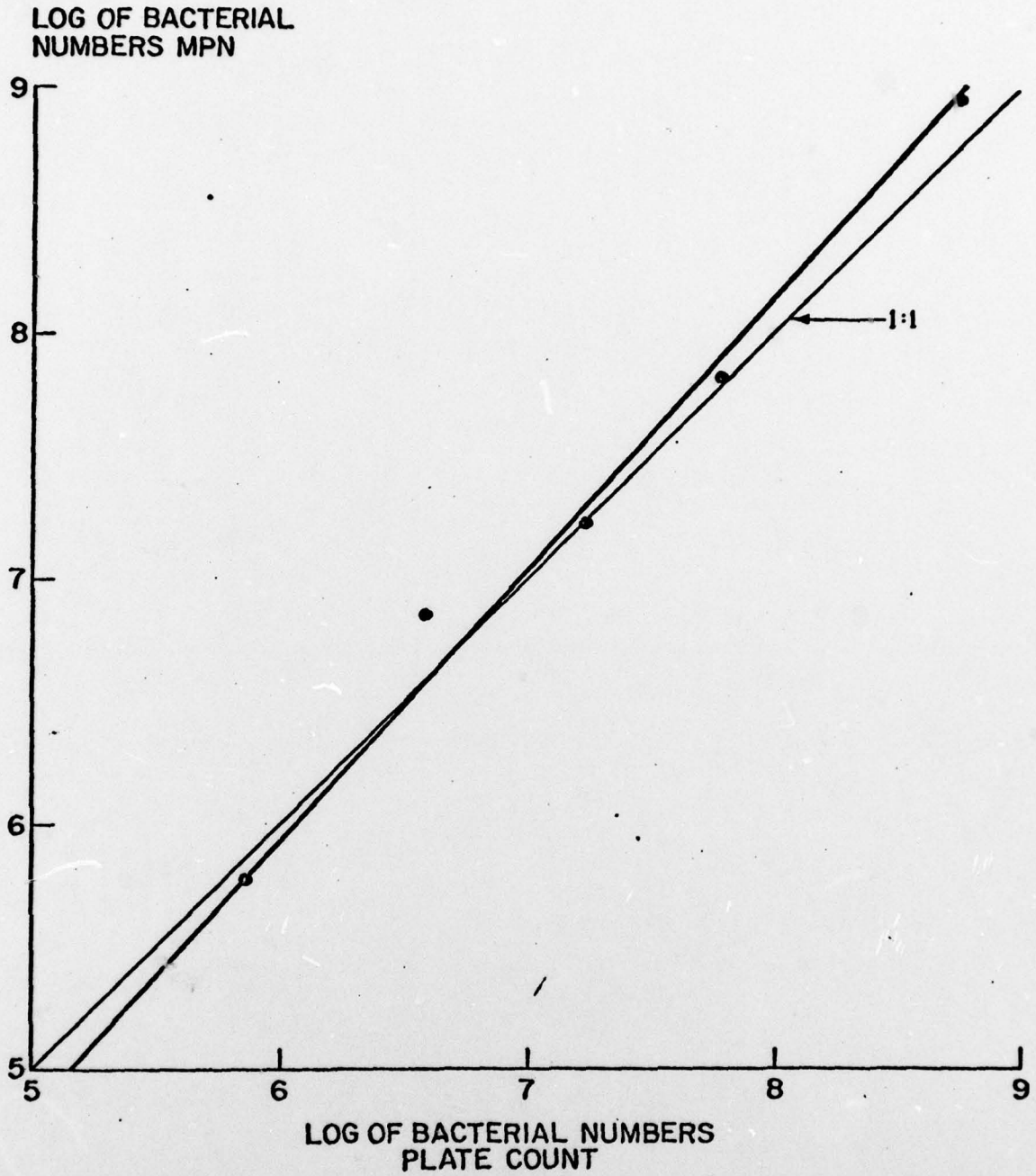
RELATIONSHIP BETWEEN THE PERCENTAGE OF FLUORESCENT DROPLETS PER FIELD OF VIEW AND THE CELL DENSITY OF E.coli DETERMINED BY PLATE COUNTS

FIGURE 7

To ensure that the rapid coliform detection method could be considered comparable to the MPN multiple-tube fermentation technique for the counting of total coliform numbers, the counts achieved by plate counts of E. coli Neotype on nutrient agar was compared to the counts in multiple fermentation tubes. Test results are shown on Figure 8. As shown thereon, there is a linear relationship between the two E. coli Neotype counting techniques.

Since it is a large step from determining the coliform numbers in cell suspensions of E. coli Neotype to the examination of "real world" samples, the rapid coliform detection method was used to count coliforms in primary-treated sewage, as well as E. coli suspensions. Numbers of coliform bacteria within a raw sewage sample and a cell suspension of E. coli Neotype were determined using the rapid coliform detection method, multiple tube fermentation and membrane filter techniques and plate counts on Eosin Methylene Blue and MacConkey's agar. Figures 9 and 10 illustrate the apparatus used for the latter techniques. The sewage sample was collected from the primary treatment tank of the Islip Salvage Plant, Babylon, New York. Suspended solids were removed by vacuum filtration through a Whatman #1 filter and centrifuged at 500 g for ten minutes. The sample was then processed using standard techniques for total coliform determinations.

A comparison of different coliform-counting techniques was made using a sewage sample and a cell suspension of E. coli Neotype as a source of coliform bacteria. The techniques employed were the plate counts on Eosin Methylene Blue and MacConkey's agar, the MPN determination using the multiple-fermentation tube technique and the membrane filter technique. The results are summarized in Table 1. The rapid detection method overestimated the number of coliforms in the sewage sample and underestimated the number in the E. coli suspension, compared to the other techniques. A possible reason for the higher coliform numbers with the rapid detection method is the presence of B-galactosidase-positive organisms within the sewage that do not grow on the selective media used in the other techniques. In contrast with an E. coli suspension, repair mechanisms may operate with the plating and multiple-fermentation tube techniques that do not occur in the rapid coliform detection method. However, no conclusion about the correlation between the five techniques can be established on the basis of two determinations. The results appear to lie within the error inherent in these techniques.



CORRELATION OF COLIFORM CELL DENSITIES DETERMINED BY MPN AND PLATE COUNT METHODS

FIGURE 8

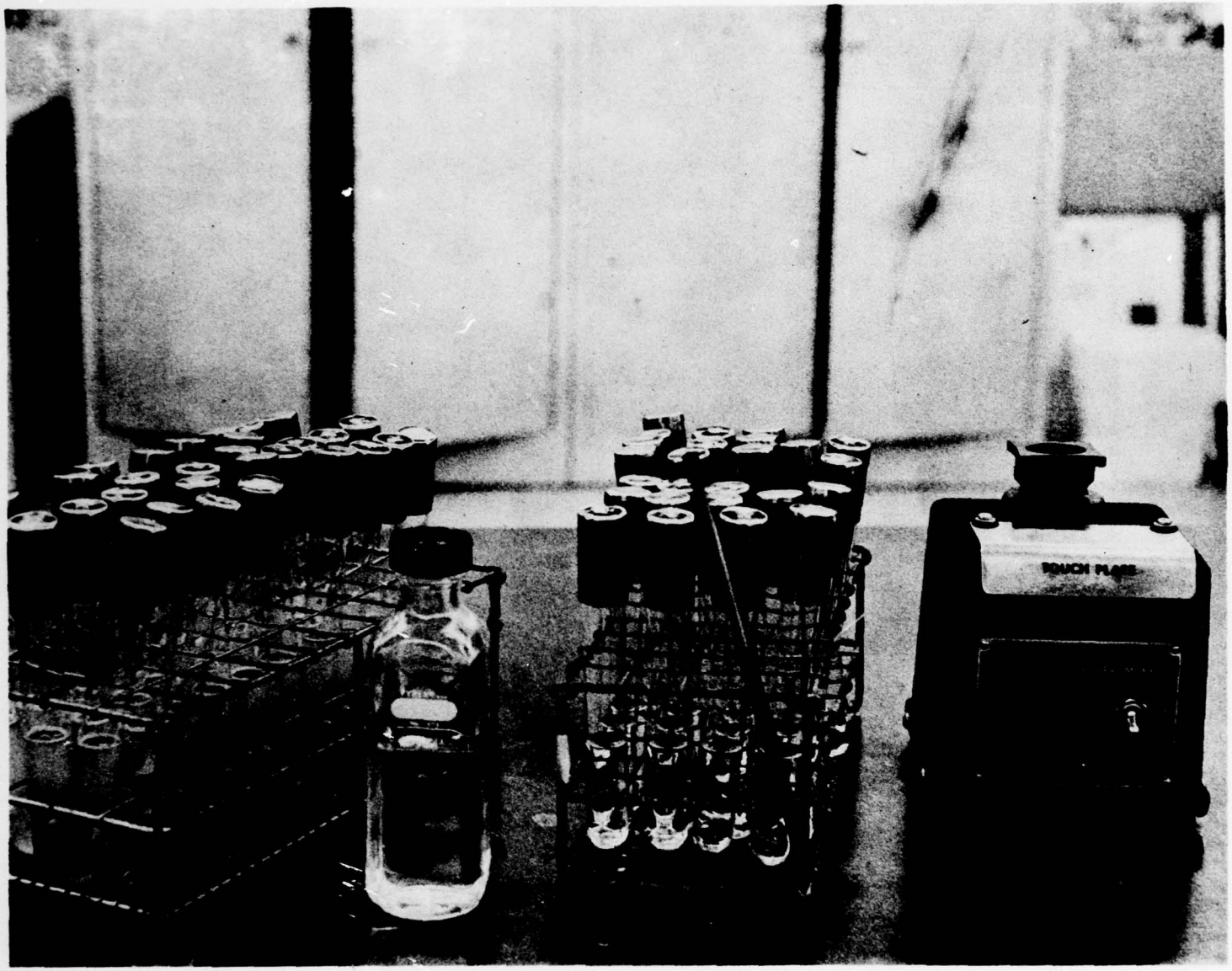


FIGURE 9 - PHOTOGRAPH OF MULTIPLE-TUBE FERMENTATION SETUP

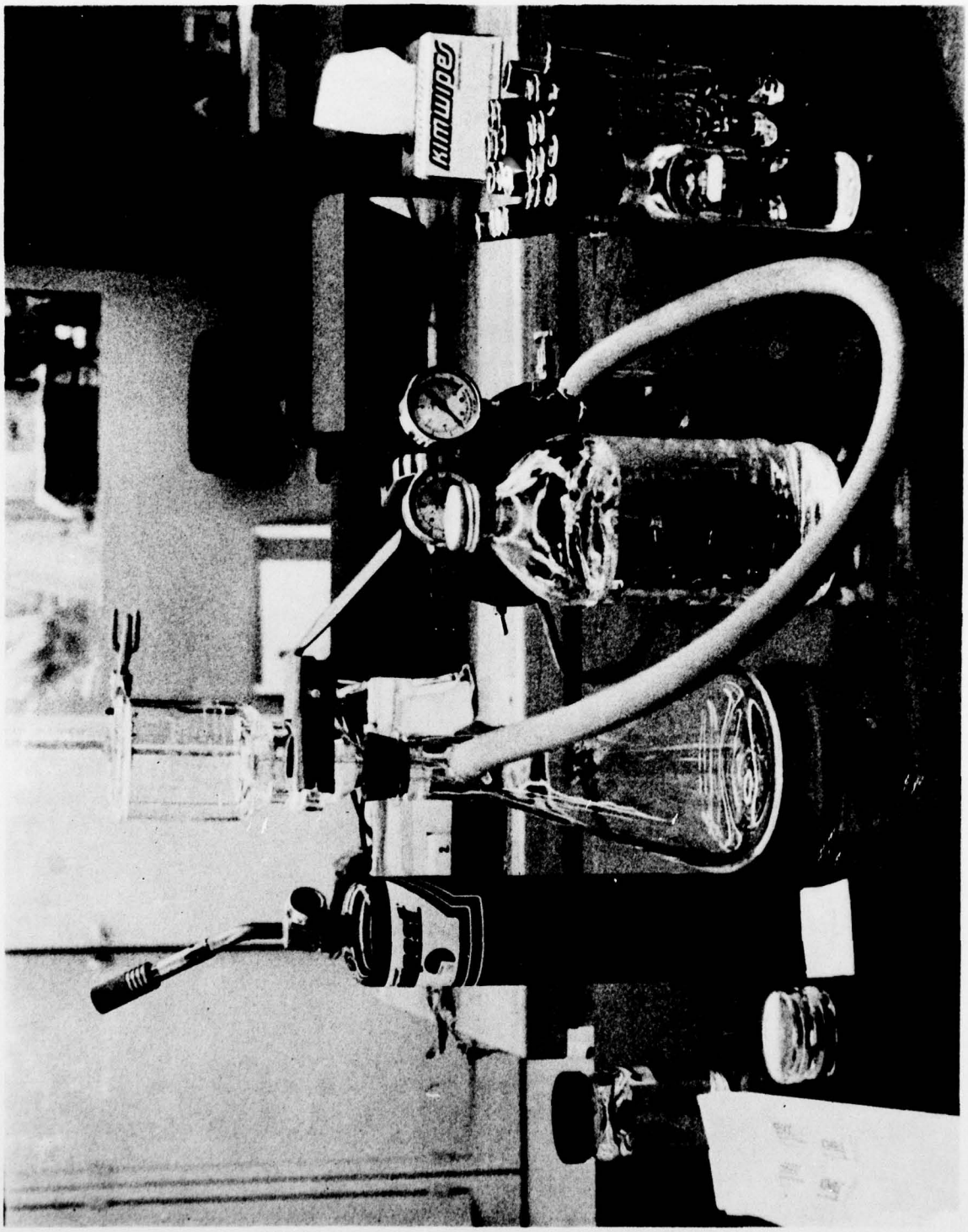


FIGURE 10 - PHOTOGRAPH OF MEMBRANE FILTRATION TEST APPARATUS

TABLE 1

Comparison of different coliform counting techniques.

Technique	Coliform Counts (organisms per ml)	
	Primary Sewage Sample	*E. coli* Neotype
Plate counts a) MacConkey's agar	3.2×10^6	1.5×10^8
b) Eosin Methylene Blue agar	3.4×10^6	1.3×10^8
MPN	2.5×10^5	1.8×10^8
Membrane filtration	6.0×10^4	1.3×10^8
Rapid detection	8.2×10^6	6.5×10^7

During experimentation to construct a standard curve of the relationship between the percentage of fluorescent droplets per field of view for coliform suspensions containing 10^8 and 10^9 organisms per ml and plate counts of the number of organisms in those suspensions, a series of coliform suspensions of unknown cell density were included in the runs. Data on the level of agreement between the two techniques are contained in Table 2.

Table 2

Coliform Suspension of Unknown Cell Density
Included in the Rapid Coliform Detection Series

	Plate Count (Organisms /ml)	Percentage Fluorescent droplets	Standard Deviation	Rapid Coliform Detection Method Count (Organisms /ml)	Percentage Disagreement
	1.3×10^8	68.3	10.6	8.3×10^7	36
	8.8×10^7	72.7	8.0	9.5×10^7	8
	5.0×10^7	58.9	11.6	6.0×10^7	20
	2.8×10^7	48.5	8.8	3.3×10^7	18
	2.8×10^6	13.2	4.7	2.7×10^6	4
	1.4×10^6	8.4	4.5	1.3×10^6	7
	9.5×10^5	5.3	3.0	9.5×10^5	0
Mean	4.3×10^7	39.3	7.3	3.9×10^7	13

Concentration of Bacteria

The preceding test results indicate that the fluorescence approach can indeed detect and quantify bacteria in the $>10^5$ cells per ml level in less than 2 hours. Although the test procedure can detect a single bacterium, it becomes a problem of statistics in finding 2 bacteria in 1 ml of water, if we are to meet the detection level required by EPA regulations. Two methods have been proposed to meet the problem of quantifying the fluorescence technique at the 2 per ml concentration level. The first method involves concentration to the $> 10^5$ level, using centrifugation. The second method involves automatic scanning of a one (1) ml sample. Only the first approach has been investigated under this contract.

Concentration by Centrifugation

Coliform suspension, with numbers lower than 10^6 organisms per ml, can be concentrated by a novel centrifugation technique. The technique involves the addition of cells of the organism Staphylococcus epidermidis to the coliform suspensions. (Optical density 0.6, final concentration $>10^8$ cells per ml.) The mixed cell populations are then harvested, as a firm bacterial pellet, by centrifugation (12,000 g for ten minutes at 8°C). Pellets are recovered by being cut out from thin-walled polycarbonate centrifuge tubes and resuspended in minimal volumes of buffer, to achieve the maximum concentration.

The co-centrifugation of E. coli Neotype with Staphylococcus epidermidis as described above, proved to be a viable procedure for the concentration of coliform bacteria to a sufficient cell density (10^6 organisms per ml) to be counted by the rapid coliform detection method. Details of three concentration experiments are outlined in Table 3. [Concentration and percentage recovery was determined by plate counts on selective media like MacConkey's agar which permit the growth of E. coli but not Staph. epidermidis.]

TABLE 3

Concentration of *E. coli* Neotype Using

Co-centrifugation with *Staphylococcus epidermidis*

Theoretical Concentration	Cell Density (Organisms /ml) Initial	Final	Actual Concentration	Percent Recovery
5x	1.3×10^4	6.4×10^4	4.9x	98%
80x	1.4×10^4	7.9×10^5	75.9x	97%
1000x	1.0×10^3	4.2×10^6	4200x	384%

These results indicate that centrifugation may be a feasible procedure for the concentration of coliform bacteria for the rapid coliform detection method, at least down to the 10^3 coliforms per ml range.

If co-centrifugation of the coliform bacteria with excess numbers of *Staphylococcus epidermidis* is to be used as a concentration technique, it had to be shown that the presence of *Staph. epidermidis* in the droplets did not interfere with visualization of fluorescence. To this end, increasing concentrations of *Staph. epidermidis* were added to a coliform suspension and the effect on the number of fluorescent droplets determined. Results shown on Table 4 indicate that large excesses of a bacterium without B-D-galactosidase activity within the droplets do not invalidate the rapid coliform detection procedure.

TABLE 4

Effect of Increasing *Staphylococcus epidermidis* Concentration of the Percentage of Fluorescent Droplets Derived from a Coliform Suspension (5×10^6 organisms per ml).

Optical Density of *Staph. epidermidis* (0.5 OD ≡ 2 x 108 organisms per ml)	Percentage of Fluorescent Droplets	Standard Deviation
Lactate medium only	18.5	6.3
0.1	21.5	6.9
0.3	17.2	6.1
0.5	28.2	7.3
0.7	19.5	7.2
Mean	21.0	6.8

SUMMARY AND CONCLUSIONS

The experimental program has verified the test data of Rotman in that single coliform bacteria, contained in microdroplets, can be visually detected on a microscope slide, in a background of non coliform bacteria. Further, we have demonstrated that visual detection using the fluorescence technique, can be made in < 1.5 hours. We have also demonstrated that coliforms, in the 10^5 to 10^9 bacterial/ml range can be quantified.

Methods to reduce the quantification level below 10^5 bacteria/ml have been briefly examined experimentally. Emphasis was placed on sample concentration as the preferred method of lowering the quantification level. Filtration, using microporous filters for sample concentration, was found to be unreliable. Co-centrifugation using Staphylococcus epidermidis, was found to be promising, at least to the 100x concentration level.

Most of the experimental effort was conducted using E. coli Neotype, the coliform strain believed to be most representative of the coliform class of bacteria. A brief series of tests, using primary treated sewage samples, indicated that the fluorescence method may be less succeptable to coliform counting errors than conventional methods.

Based upon the test results presented herein and certain preliminary concepts presented to cognizant contract personnel, it is our conclusion that (1) quantification can be reduced to the 2 coliforms per ml level specified by the EPA and (2) the technique can be modified to reduce detection and quantification time to less than one hour.

REFERENCES

1. Anon., Definition of Terms Concerning Coliform Bacteria and Recommended Methods for their Detection, N.Z.J. Sci. 19:215-219, (1976).

2. Metcalf, T. G., Indicators for Viruses in Natural Waters. In Water Pollution Microbiology Volume II Edited R. Mitchell, John Wiley & Sons, (1978).

3. American Public Health Association, Standard Method for the Examination of Water and Wastewater. 14th Edition, (1975).

4. Pardee, A. B., F. Jacob and J. Monod, The Genetic Control and Cytoplasmic Expression of "Inducibility" in the Synthesis of B-galactosidase by E. coli J. Mol. Biol. 1:165-178, (1959).

5. Rotman, B., Measurement of Activity of Single Molecules of B-D-galactosidase. Proc. Nat. Acad. Sci. 47:1981-1991, (1961).

APPENDIX Statistical Analyses of the Coliform Data

Coliforms per ml x 10^6 (X)			490	380	250	160	80
Percent Fluorescent droplets (Y)			94.7	80	95.4	77.0	74.2
73	69	50	47	13	12	10	9.4
49.3	73.2	58.5	63.0	45.0	49.9	29.0	16.3
8.5	3.7	2.6	2.1	1.8	1.7	0.6	0.5
20.7	16.3	14.4	12.8	6.9	4.0	4.3	2.9
0.4	0.3	0.3	0.1				
1.4	1.2	1.0	0.6				

$\Sigma X = 1666.0$ $\Sigma Y = 892.3$

$\bar{X} = 66.6$ $\bar{Y} = 35.7$ $n = 25$

$\Sigma X^2 = 494404.6$ $\Sigma Y^2 = 57914.3$ $\Sigma XY = 135511.0$

$(\Sigma X)^2/n = 111022.2$ $(\Sigma Y)^2/n = 31848.0$ $(\Sigma X)(\Sigma Y)/n = 59248.7$

$\Sigma x^2 = 383382.4$ $\Sigma y^2 = 26066.3$ $\Sigma xy = 76262.3$

(Continued Page 29)

APPENDIX (Continued)

a) Line of best fit

$$b = \Sigma xy/\Sigma y^2 = 76262.3/26066.3 = 2.93$$

$$\hat{y} = 66.6 + 2.93\ (x-35.7)$$

$$y = 2.93x - 38.0$$

b) Estimation of the goodness of fit of line on the data

$$\Sigma dy.x^2 = \Sigma y^2 - (\Sigma xy)^2/\Sigma x^2$$

$$= 57914.3 - (7.63 \times 10^4)^2/3.83 \times 10^5$$

$$= 57914.3 - 15200.0$$

$$= 42714.0$$

$$Sy.x^2 = \Sigma dy.x^2/(n-2) = 42714.0/23$$

$$= 1857.1$$

$$Sy.x = \sqrt{1857.1} = 43.1$$

$$S_b = Sy.x/\sqrt{\Sigma x^2} = 43.1/619.2$$

$$= 0.07$$

$$t = b/s_b = 0.20/0.07 = 2.86$$

$$df = n - 2 = 23$$

(Continued Page 30)

APPENDIX (Continued)

c) Correlation Coefficient

to estimate the degree of closeness of the linear relationship between Y and X

$$r = \frac{\Sigma xy}{\sqrt{(\Sigma x^2)(\Sigma y^2)}} = \frac{76262.3}{\sqrt{(383482.4)(26066.3)}}$$

$$= \frac{76262.3}{99975.0}$$

$$= 0.76$$